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The Amino Acid Sequence of an Extracellular Nuclease of *Staphylococcus aureus*

II. THE AMINO ACID SEQUENCES OF TRYPTIC AND CHYMOTRYPTIC PEPTIDES

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SUMMARY

Treatment of the extracellular nuclease of *Staphylococcus aureus*, strain V8, with cyanogen bromide was previously shown to produce five polypeptides which could be arranged in a linear order. Trypsin digestion of these yielded sets of peptides, in each of which the carboxyl-terminal fragment could be identified by the absence of lysine or arginine, or by the presence of homoserine. The amino acid sequence of each tryptic peptide has been determined. These results, together with the isolation and characterization of peptides produced by chymotrypsin digestion of the intact nuclease, account closely for the amino acid composition of the cyanogen bromide fragments.

The linear arrangement of the five peptide fragments produced by cyanogen bromide treatment of the extracellular nuclease of *Staphylococcus aureus*, strain V8, has been described (1, 2). Peptides produced by tryptic digestion of each cyanogen bromide fragment were also separated and subjected to amino acid analyses (2). The present paper describes the isolation and sequence determination of trypsin fragments produced by digestion of the intact nuclease and of the cyanogen bromide fragments. These peptides account for the total amino acid content of the protein. Peptides produced by chymotryptic digestion have also been separated and analyzed. The combined results of the previous and present studies permit the alignment of all of the trypsin fragments within each cyanogen bromide fragment. The reconstruction of the entire sequence of the nuclease is presented in the succeeding paper in this volume.

EXPERIMENTAL PROCEDURE

Unless otherwise specified, the materials and procedures used were those presented in a previous report (2).

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Chromatography of Tryptic Fragments of Nuclease—Tryptic digestion of the nuclease was performed with approximately 2 μmoles of the protein, and the fragments obtained were fractionated on a Dowex 50 column (1 × 94 cm) as previously described (2). The elution pattern is shown in Fig. 1. Fractions comprising a peak were pooled and examined for purity by two-dimensional peptide mapping (2). Further purification, when necessary, was performed on Whatman No. 3MM paper as reported previously (2).

Chromatography of Chymotryptic Fragments of Nuclease—Chymotryptic digestion of 6 μmoles of nuclease in 5 ml of H_2O was carried out with 2 mg of α-chymotrypsin (Worthington, three times crystallized). The pH of the mixture was maintained at 8 with 1 M NH_4OH during the large scale incubation, which was carried out at 37° for 1 hour. The extent of chymotryptic cleavage was checked, in preliminary experiments, by two-dimensional peptide mapping of aliquots withdrawn after 0, 20, 60, and 240 min. After 60 min, no residual undigested "core" remained. The digestion mixture was lyophilized, and the dried sample was subjected to chromatographic fractionation (Fig. 2) on a Dowex 50 column (1 × 94 cm) as described previously (2). When necessary, further purification was performed by paper electrophoresis, paper chromatography, or two-dimensional peptide mapping as described in the previous report (2).

Digestions with Peptidases—Five milligrams of leucine aminopeptidase (Worthington) were dissolved in 2 ml of 0.05 M NH_4HCO_3 containing 0.01 M $MgCl_2$ and dialyzed against 3 liters of the same buffer at 4° for 48 hours with three changes. The dialyzed protein solution was incubated at 37–40° for 2 hours with magnesium ions to activate the enzyme and was stored at −20°.[1] The leucine aminopeptidase digestion mixture consisted of 0.05 to 0.2 μmole of peptide and the peptidase, at a level of 2 to 10% by weight of the substrate, in 0.2 to 0.5 ml of 0.05 M NH_4HCO_3-0.01 M $MgCl_2$ buffer, pH 8. Incubations were performed at 37° for 5 to 6 hours, unless otherwise specified.

[1] A similar preparation made from diisopropyl fluorophosphate-treated leucine aminopeptidase (Worthington) was provided by Dr. Francesco DeLorenzo and used for some of the digestions.

Purified carboxypeptidase B was donated by Dr. S. Korenman (3). Incubations with carboxypeptidase B were performed as with carboxypeptidase A (2).

Pronase, obtained from Calbiochem (Grade B), was prepared as a 0.3% solution in 0.05 M NH_4HCO_3 containing 0.001 M $CaCl_2$ (4). The incubation mixtures contained 0.1 ml of 0.05 M NH_4HCO_3 (pH 8), 0.05 to 0.1 μmole of peptide, and 5 μl of Pronase solution. Incubations were performed at 37° for 16 hours.

The amino acid analyses of the peptidase digests were carried out as described previously (2). Blanks, containing approximately 50 μg of each peptidase, were incubated without substrate at 37° for 20 hours. The amounts of free amino acids (mainly serine, glutamic acid, and glycine) in these blank incubations were less than 0.003 μmole.

Dilute Acid Hydrolysis of Peptides—Partial acid hydrolysis of peptides containing aspartic acid or asparagine was performed by the method of Tsung and Fraenkel-Conrat (5). Peptides (0.05 to 0.2 μmole) were incubated in 0.2 to 0.5 ml of 0.03 N HCl in sealed, evacuated tubes at 110° for 15 hours and taken to dryness under reduced pressure over NaOH. The dried material was dissolved in 0.1 to 0.2 ml of H_2O. Aliquots (containing 0.02 to 0.04 μmole of the original peptide) were removed for determination of free amino acids on the amino acid analyzer, and the production of peptide fragments was monitored by two-dimensional peptide mapping. Free aspartic acid could also be estimated qualitatively on the peptide maps. Peptide fragments were purified by paper electrophoresis, paper chromatography, or two-dimensional peptide mapping as described previously (2).

Hydrazinolysis—The hydrazinolysis of peptides was performed by the method of Winstead and Wold (6) as reported by Korenman, Craven, and Anfinsen (3). The dried peptide (0.02 to 0.04 μmole) was incubated with 0.5 ml of anhydrous hydrazine in an evacuated, sealed tube at 110° for 10 hours. After removal of excess hydrazine under reduced pressure over concentrated H_2SO_4, the sample was applied on the amino acid analyzer without previous extraction of hydrazides.

Amino Acid Analyses—Values for amino acids are expressed in micromoles. Analyses were performed by the method of Spackman, Moore, and Stein (7) as reported previously (2). Values for amino acids present in amounts less than 0.003 μmole are not included unless otherwise specified. When it is necessary to present values of less than 0.003 μmole, the observed values of all other amino acids in the peptide are also included. Asparagine and glutamine released by the peptidase digestions were determined quantitatively on the amino acid analyzer, and qualitatively by paper chromatography as described previously (2).

Electrophoretic Mobility—Paper electrophoresis on Whatman No. 3MM paper was performed at pH 6.5 (8), at 46 volts per cm for 60 min, to examine the electrophoretic mobilities of peptides and amino acids (released from peptides with peptidase digestion). Known neutral, basic, and acidic amino acids served as standards. The mobilities of the samples are presented, qualitatively, as "basic," "acidic," or "neutral."

RESULTS

Tryptic and Chymotryptic Peptides of Nuclease

Tryptic Peptides—The amino acid compositions of tryptic fragments purified from the chromatographic fractions (Fig. 1) are presented in Table I, together with their electrophoretic mobilities at pH 6.5.

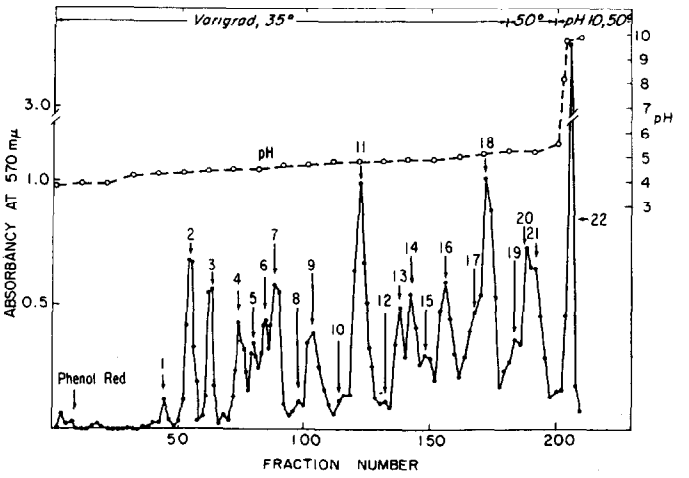

FIG. 1. Chromatography of tryptic peptides of the nuclease. Experimental details are given in "Experimental Procedure." Phenol red was added to the sample as an indicator of the elution front. The pooled fractions comprising the peaks and the valleys are indicated by the *arrows*. These fractions are designated T-1, T-2, etc., in the text.

Another tryptic digest of the nuclease was separated by two-dimensional peptide mapping on four or more sheets of Whatman No. 3MM paper, and the amino acid compositions of the eluted peptides were determined as described previously (2). No peptides were detected in the eluates from the preparative maps that were not also found in the fractions prepared by ion exchange chromatography (Table II). Furthermore, peptides isolated from the tryptic digests of the intact nuclease were completely consistent with those derived from the cyanogen bromide fragments, except of course for those peptides in the latter digests that were derived from portions of the sequence containing methionine. Thus, Peptides T-7b, T-9d, T-15b, and T-17a (or F15, F17, F18, and F19; see Reference 2) were not found after cyanogen bromide treatment. Peptide T-11a could not be clearly located on the peptide maps, presumably because this peptide did not form a discrete, uncontaminated spot.

The counterpart of Peptide T-18a (or F9) was not found in the set derived from the cyanogen bromide fragments. However, Peptide T-V-7b was shown to be a part of Peptide T-18a and Peptide T-11b was the sum of Peptide T-V-6, threonine, and lysine, as described below.

Minor Components of Tryptic Peptide Mixture—Further purification and amino acid analyses were performed on minor components of the tryptic digests of the cyanogen bromide fragments (2). Cyanogen bromide Fragments A and C were separated in fairly pure form on Sephadex G-50, but Fragments D and E were contaminated with small amounts of Fragments A and C, respectively (2). A few tryptic peptides, derived from the contaminated fragments, were separated as minor components (2). Peptides T-V-6 and T-V-13, were previously assigned to CNBr Fragment C (2). Peptide T-V-6 was found, in the present studies, to be identical with a product of cleavage of the minor component, T-III-8a,[2] by the intrinsic chymotrypsin-

[2] Lys, 0.016(1); Thr, 0.018(1); Ser, 0.018(1); Glu, 0.021(1); Pro, 0.019(1); Gly, 0.022(1); Ala, 0.041(2); Tyr, 0.017 (1); Phe, 0.033(1); yield, 4%. R_{FC}, 0.40; R_{FE}, 0.42 (see Peptide F_{21} in Table II).

TABLE I

Amino acid composition of tryptic fragments of nuclease V8

No half-cystine was found in any of the peptides analyzed. When peptides from the chromatographic fractions (see Fig. 1) were purified further, the purification methods (paper electrophoresis, paper chromatography, and two-dimensional peptide mapping) are indicated as E, C, and M, respectively. The yields were calculated from the amino acid analyses of aliquots of the chromatographic fractions. When more than one peptide was contained in the same fraction, the yield of each peptide was calculated on the basis of amino acid residues specific to each peptide. Special colors produced by cadmium ninhydrin staining (2) are indicated. The electrophoretic mobilities at pH 6.5 were examined with aliquots of the chromatographic fractions unless otherwise specified. Tryptophan was not determined, except in Peptide T-2. With this peptide leucine aminopeptidase digestion released free tryptophan in a 1:1 molar ratio to leucine. Fraction 22 accounted for several spots on the peptide map. The amino acid analyses of the eluted map components indicated that these were large fragments, present in small amounts, that overlapped other peptide fragments.

Amino acid	T-2	T-3	T-4a	T-4b	T-5a	T-5b	T-6b	T-6c	T-7b	T-7c	T-7d	T-9a	T-9b	T-9d
Lysine		0.045(1)		0.004(1)	0.008(1)		0.007(1)	0.084(1)	0.025(1)[a]	0.022(1)	0.060(1)	0.013(1)[a]	0.006(1)	
Histidine														0.008(1)
Arginine														
Aspartic acid	0.158(4)	0.087(2)		0.005(1)					0.042(1)				0.011(1)	0.015(1)
Threonine		0.040(1)		0.007(2)	0.007(1)						0.142(2)			
Serine	0.063(2)		0.007(1)								0.061(1)			
Glutamic acid	0.088(2)		0.007(1)	0.006(1)	0.005(1)		0.008(1)		0.042(1)	0.026(1)	0.005(0)	0.026(1)		0.015(1)
Proline			0.009(1)	0.005(1)	0.006(1)									
Glycine	0.042(1)	0.044(1)	0.006(1)			0.007(0)			0.008(0)	0.011(1)[a]	0.008(0)	0.030(1)	0.012(1)	0.005(0)
Alanine	0.042(1)	0.040(1)	0.010(1)	0.003(0)	0.007(1)	0.013(1)			0.047(1)		0.039(1)[a]	0.031(1)	0.011(1)	0.015(1)
Valine		0.041(1)		0.006(1)		0.012(1)			0.056(1)	0.021(1)				0.032(2)
Methionine									0.010(1)[a,b]					0.002(1)[a]
Isoleucine	0.032(1)	0.041(1)			0.007(1)								0.007(1)	
Leucine	0.035(1)			0.011(3)[c]	0.006(1)							0.033(1)		0.015(1)
Tyrosine			0.002(1)[d]			0.000(1)[d]							0.008(1)	
Phenylalanine			0.003(1)											
Electrophoretic mobility	Acidic	Acidic	Acidic	Neutral[e]	Neutral	Neutral	Neutral	Basic	Neutral	Neutral Yellow	Basic	Neutral[f]	Neutral	Neutral
Ninhydrin color														
Purification method			E	E	M	M	E	E	M	M	M	M	M	M
Yield	82%	100%	32%	92%	37%	44%	63%		87%	85%	85%	83%	40%	90%

Amino acid	T-11a	T-11b	T-11c	T-11d	T-13a	T-13c	T-14a	T-15b	T-16d	T-17a	T-17d	T-18a	T-18b	T-20
Lysine	0.021(2)	0.045(1)	0.118(2)		0.018(2)	0.031(2)	0.056(1)		0.028(1)[a]	0.012(1)	0.015(1)	0.021(2)		0.026(1)
Histidine											0.009(1)[c]	0.013(1)		0.020(1)
Arginine				0.021(1)				0.016(1)	0.046(1)				0.026(1)	0.029(1)
Aspartic acid	0.014(1)						0.065(1)		0.035(1)					0.041(2)
Threonine	0.005(0)	0.042(1)						0.016(1)	0.016(1)[a]			0.014(1)		0.022(1)
Serine	0.005(0)	0.032(1)	0.060(1)											
Glutamic acid	0.030(2)	0.060(1)	0.145(2)	0.034(1)	0.015(1)	0.024(1)		0.021(1)	0.010(0)			0.016(1)		0.055(2)
Proline		0.060(1)						0.017(1)			0.016(1)	0.015(1)		0.026(1)
Glycine	0.010(0)	0.047(1)		0.022(1)	0.012(1)		0.123(2)	0.013(1)	0.033(1)		0.013(0)[c]		0.016(1)	
Alanine	0.008(0)	0.092(2)	0.152(2)				0.127(2)					0.015(1)[g]		
Valine	0.014(1)				0.011(1)									0.035(1)
Methionine								0.008(1)[h]		0.004(1)				
Isoleucine	0.012(1)						0.058(1)					0.013(1)[g]		
Leucine	0.004(0)						0.062(1)			0.008(1)		0.022(2)[g]		0.050(2)
Tyrosine		0.000(1)[d]					0.017(2)[d]		0.019(1)[d]	0.010(1)			0.008(1)	0.022(1)
Phenylalanine	0.014(1)	0.051(1)						0.016(1)						
Electrophoretic mobility	Neutral	Neutral	Basic	Basic Yellow	Basic	Basic	Neutral Yellow	Basic	Basic Orange	Basic	Basic	Basic	Basic	Basic
Ninhydrin color														
Purification method	C	C	C	C	E	E	E	E	M	E	E	E	E	
Yield	30%	73%	40%	92%	50%	55%	55%	30%	78%	48%	100%	82%	57%	100%

[a] The compositions of certain peptides eluted from peptide maps after light staining with ninhydrin indicate partial destruction of NH_2-terminal and lysine residues (*e.g.* T-16d, which contains NH_2-terminal threonine).

[b] The parent fraction contained methionine in an amount comparable to other residues.

[c] See the text and Table II.

[d] The number of residues of tyrosine was estimated from the amino acid analyses of the parent chromatographic fractions (2).

[e] The mobility was only tentatively judged as neutral because of the tailing of the spot.

[f] Subsequent degradations with leucine aminopeptidase and the Edman method indicate the sequence of this peptide to be Gln-Gly-Leu-Ala-Lys. The electrophoretic neutrality of Peptide T-9a may be due to formation of an NH_2-terminal pyrrolidonecarboxylic acid residue.

[g] Alanine, isoleucine, and leucine were not determined in this analysis. The values obtained with another analysis are presented. The values for threonine and glutamic acid were used for standardization.

[h] The sum of methionine (0.006) and methionine sulfone (0.003) is presented.

TABLE II

Comparison of tryptic peptides from native nuclease with those prepared from cyanogen bromide fragments of nuclease

The peptides were compared and identified on the basis of amino acid composition, positions on two-dimensional peptide maps, color by ninhydrin staining, and patterns of elution from columns of Dowex 50. The primary data are presented in Table I and Fig. 1 and in the previous report (2). The chromatographic yields from Dowex 50 columns of the tryptic peptides prepared from cyanogen bromide fragments are indicated in parentheses and were calculated as for the tryptic peptides of the native nuclease summarized in Table I.

Tryptic peptides from native nuclease		Composition	Tryptic peptides from cyanogen bromide fragments (2)	
Isolated by chromatography on Dowex 50	Isolated by peptide mapping		Peptide	Parent cyanogen bromide fragment
T-2	F_{25}	Asp_4, Ser_2, Glu_2, Gly, Ala, Ile, Leu, Trp	T-III-2 (50%)	A
T-3	F_{22}	Lys, Asp_2, Thr, Gly, Ala, Val, Ile	T-V-2 (50%)	A
T-4a	F_{26}[a]	Ser, Glu, Pro, Gly, Ala, Tyr, Phe	T-V-6 (37%)	C
T-4b	F_{23}	Lys, Asp, Thr_2, Glu, Pro, Val, Leu_3[b]	T-V-5b (25%)	C
T-5a	F_{20}	Lys, Thr, Glu, Pro, Ala, Ile, Leu	T-V-7b (22%)	A
T-5b	F_{27}[a]	Ala, Val, Tyr	T-III-4C (16%)	E
T-6b		Lys, Glu	T-III-5b (39%)	E
T-6c	F_4	Lys		
T-7b	F_{17}	Lys, Asp, Glu, Ala, Val, Met		C-D
T-7c	F_{13}	Lys, Glu, Gly, Val	T-V-8b (15%)	C
T-7d	F_{12}	Lys, Thr_2, Ser, Ala	T-V-8a (37%)	A
T-9a	F_{14}[c]	Lys, Glu, Gly, Ala, Leu	T-III-7b (46%)	E
T-9b		Lys, Asp, Gly, Ala, Ile, Tyr	T-VII-5a (42%)	D
T-9d	F_{19}	Arg, Asp, Glu, Ala, Val_2, Met, Leu		D-E
T-11a		Lys_2, Asp, Glu_2, Val, Ile, Phe	T-VII-8a (21%)	D
T-11b	F_{21}	Lys, Thr, Ser, Glu, Pro, Gly, Ala_2, Tyr, Phe		C
T-11c	F_8	Lys_2, Ser, Glu_2, Ala_2	T-III-8b (50%)	E
T-11d	F_{10}	Arg, Glu, Gly	T-VII-8b (34%)	D
T-13a	F_6	Lys_2, Glu, Gly, Val	T-V-13 (55%)	C
T-13c	F_5	Lys_2, Glu	T-III-9c (41%)	E
T-14a	F_{24}	Lys, Asp, Gly_2, Ala_2, Ile, Leu, Tyr_2	T-VII-9 (7%)	D
T-15b	F_{18}	Arg, Thr, Glu, Pro, Gly, Met, Leu, Phe		B-C
T-16d		Lys, Arg, Asp, Thr, Gly, Tyr	T-VII-10a[d]	D
T-17a	F_{15}	Lys, Met, Leu, Tyr		A-B
T-17d	F_2	His, Pro, Lys	T-V-15b[e] (30%)	C
T-18a	F_9	Lys_2, His, Thr, Glu, Pro, Ala, Ile, Leu_7		A
T-18b	F_{14}[c]	Tyr, Gly, Arg	T-VII-11a (26%)	D
T-20	F_{11}	Lys, His, Arg, Asp_2, Thr, Glu_2, Val, Leu_2, Tyr	T-III-12 (68%)	E

[a] These components, occasionally observed on peptide maps, were presumably due to instrinsic chymotryptic-like activity in the trypsin preparation (see the text).

[b] Three residues of leucine were assigned on the basis of the amino acid analysis of T-V-5b (2).

[c] Both peptides occupied essentially the same position on the peptide map (see Reference 2). The analysis of the sample obtained from Component F_{14} (see Reference 2) gave the sum of their amino acid contents.

[d] See the text.

[e] Identified on the basis of the peptide map and amino acid composition of the parent chromatographic fraction.

like activity of the trypsin preparation (9, 10). Peptide T-V-13 was identical with the minor component, T-III-9b.[3] Peptide T-V-7b (2) was previously found to contain a fraction of 1 eq of histidine. However, Peptide T-5a (Table I), which had the same amino acid composition and essentially the same position on the peptide map as Peptide T-V-7b, contained no histidine and gave a negative Pauly reaction. It was concluded, therefore, that Peptide T-V-7b does not contain histidine. Peptide T-5a, obtained as a very minor component, was found to be part of Peptide T-18a, as described below.

[3] Lys, 0.018(2); Glu, 0.013(1); Gly, 0.013(1); Val, 0.015(1); yield, 6%. R_{FC}, 0.13; R_{FE}, 0.84.

This consideration of the minor component is consistent with the assignments of the tryptic peptides to cyanogen bromide fragments listed previously (Table II) (2).

Assignment of Tryptic Peptides to Cyanogen Bromide Fragments—The peptides produced by trypsin digestion of the nuclease may, on the basis of the above observations, be assigned to cyanogen bromide Fragments A, B, C, D, and E (2) as summarized in Table II. Certain peptides in this table do not contain lysine or arginine and are the result of chymotrypsin-like activity in the trypsin preparation. Four of the peptides, containing the 4 residues of methionine in the nuclease, have been assigned as overlapping peptides that bridge cyanogen

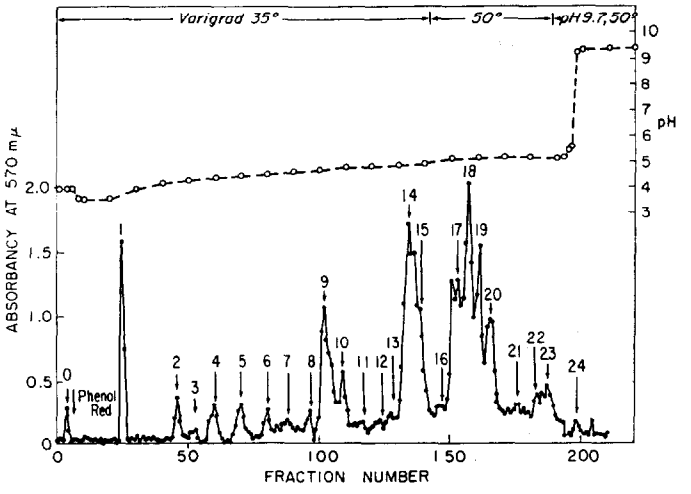

FIG. 2. Chromatography of chymotryptic peptides of the nuclease. Experimental details are given in "Experimental Procedure." The designations of the fractions are the same as described in Fig. 1, except that C-1, C-2, etc., are used in the text.

bromide fragments (2). Peptide T-18a (F9), obtained in good yield, contains Peptide T-5a (F20) and the fragment (Leu, His) Lys, which was not recovered from the chromatographic columns but has been prepared from T-18a by digestion with trypsin.

Chymotryptic Peptides—The amino acid compositions, electrophoretic mobilities at pH 6.5, and colors produced by cadmium ninhydrin of purified chymotryptic peptides obtained from the chromatographic fractions (Fig. 2) are presented in Table III. A reconstructed peptide map is shown in Fig. 3.

Presentation of Sequence Analyses

The amino acid composition of each peptide is presented in Tables II and III, unless specified otherwise. In reporting the results of Edman degradations, the recovery at each stage was calculated from the analysis of the previous stage, not on the basis of the starting material. The amino acid residue used as a basis for calculation of the recovery of residual peptide at each stage of degradation is indicated in parentheses. The eliminated residues are indicated in **boldface.** The results of peptidase digestions are presented in a similar manner. Amino acid analyses of the hydrolysates of phenylthiohydantoins are reported only qualitatively. The specificities of proteolytic enzymes used appeared to agree throughout with general experience (11). Peptide bonds involving proline were not susceptible to any of the enzymes used except Pronase (12), which was able occasionally to cleave the peptide bond involving the imino group of proline, as has been observed with pepsin (13). When peptides were fragmented by proteolytic or acid hydrolysis, the resulting fragments were purified on Whatman No. 3MM paper by two-dimensional mapping, chromatography, or electrophoresis as described previously (2). The positions on two-dimensional peptide maps of such fragments are indicated in parentheses by R_F values in the chromatographic direction (R_{FC}) and the electrophoretic direction (R_{FE}), with phenol red and lysine as standards, respectively (2). Free lysine moved 33 cm, at pH 3.6 and 46 volts per cm, in 70 min (2). No correction was made for movement due to electroosmosis. The R_F values were reproducible to approximately 10%. When the presence or absence of an amide group was uncertain, the available evidence is presented in the text, and the questionable (amide) residue is enclosed within parentheses in the sequence (see Footnote 5). Dinitrophenyl end groups, which were reported previously (2), are listed with each peptide unless otherwise specified.

Amino Acid Sequence of Tryptic Peptides

The analyses of sequences were performed either on the tryptic peptides obtained from the cyanogen bromide fragments described in a previous report (2) or on those derived from the intact nuclease. Consideration of the peptides is in accordance with the linear order of the peptides in the nuclease chain, the complete structure of which is presented in the following paper.

T-V-8a: Ala–Thr–Ser–Thr–Lys (Residues 1 to 5)—

Dinitrophenyl end group: Ala.

Edman degradation:

Stage 1 (91%): **Ala, 0.001;** Thr, 0.014(2); (Ser), 0.007(1); Lys, 0.007(1).

Stage 2 (85%): Ala, 0.001; **Thr, 0.010(1);** (Ser), 0.006(1); Lys, 0.012(1).

Carboxypeptidase B (2 hours): Lys, 0.017; Ser, 0.003.

Carboxypeptidase A (1 hour): Lys, 0.016; Thr, 0.009; Ser, 0.002.

Carboxypeptidase A (2 hours): Lys, 0.022; Thr, 0.012; Ser, 0.003. (Carboxypeptidase A was added after carboxypeptidase B.)

Carboxypeptidase B released only free lysine. A broad peak at the serine position was observed in this analysis. The amount of material in the serine position remained essentially constant after the addition of carboxypeptidase A, as did the quantity of lysine, while threonine appeared at more than half the level of lysine. These observations suggest that the peak at the serine position was not due to serine itself but probably to a peptide fragment. Neither the undigested peptide nor a blank of carboxypeptidase B showed any significant amounts of free amino acids.

T-V-7b: Glu–Pro–Ala–Thr (Leu, Ile)–Lys (Residues 10 to 16)—

Dinitrophenyl end group: Glu.

This peptide, because of its availability in only small amounts, was not used for subtractive Edman degradation. However, the phenylthiohydantoins of glutamic acid, proline, and alanine were shown to be released after the first, second, and third stages of the degradation, respectively.

Edman degradation (phenylthiohydantoin hydrolysis), three cycles: Stage 1; Glu; Stage 2, Pro; Stage 3, Ala.

Carboxypeptidase B: Lys, 0.019; Ala, Ile, Leu < 0.002.

Carboxypeptidase B and A (5 min): Lys, 0.007; Ile, 0.007; Leu, 0.007; Thr, 0.002; Ala,[4] 0.004.

Carboxypeptidase B and A (30 min): Lys, 0.020; Ile, 0.014; Leu, 0.018; Thr, 0.016; Ala,[4] 0.010.

This portion of the nuclease sequence was further established by other studies given below. The presence of a free carboxyl group on the glutamic acid residue was indicated by the electrophoretic neutrality of the peptide.

[4] This digestion was performed with chromatographic Fraction T-5, which contained Peptides T-5a (T-V-7b) and T-5b (Val–Ala–Tyr) (see Table II). Released alanine was derived from Peptide T-5b together with small amounts of valine and tyrosine.

TABLE III

Amino acid composition of chymotryptic peptides

The chromatographic yield of each peptide was calculated from the amino acid analysis of an aliquot of the fraction obtained by chromatography on Dowex 50 (see Fig. 2) as described in Table I. The purification method, the electrophoretic mobility, and the color produced by cadmium ninhydrin staining are indicated as in Table I. No half-cystine was found in any of the components. Partial destruction of NH_2-terminal residues, lysine, and tyrosine occurred with some peptides as the result of ninhydrin staining, elution, and acid hydrolysis. This is indicated by asterisks. Assignment of residue numbers was qualitative with some peptides. The number listed are based on subsequently confirmed sequences (see the text).

Amino acid	C-0	C-1	C-2	C-4	C-5d	C-5e	C-5g	C-6a	C-6b	C-7e	C-9c	C-9d	C-10b	C-14a
Lysine					0.012(1)	0.014(1)			0.014(1)		0.151(2)	0.016(1)	0.006(1)	0.026(1)
Histidine														0.020(1)[a]
Arginine													0.005(1)	
Aspartic acid	0.038(3)	0.062(3)			0.011(1)	0.029(2)	0.003(0)				0.141(2)		0.014(2)	0.043(2)
Threonine				0.048(1)					0.030(2)		0.064(1)			0.022(1)
Serine	0.022(2)	0.037(2)	0.021(1)				0.003(0)		0.015(1)					
Glutamic acid	0.028(2)	0.043(2)	0.028(1)			0.015(1)						0.029(1)	0.013(2)	0.048(2)
Proline			0.028(1)									0.031(1)		0.047(2)
Glycine	0.016(1)	0.019(1)	0.026(1)		0.015(1)	0.018(1)	0.010(0)			0.005(0)	0.073(1)	0.028(1)	0.012(2)	
Alanine	0.014(1)	0.023(1)	0.028(1)		0.012(1)	0.025(2)	0.042(1)		0.016(1)		0.090(1)		0.011(2)	
Valine						*0.015(1)*		0.039(1)			*0.082(1)*		0.012(2)	
Methionine					0.007(1)[b]	0.010(1)				0.020(1)		0.021(1)	0.004(1)	
Isoleucine										0.008(0)	0.140(2)			
Leucine						0.018(1)					0.097(1)		0.014(2)	0.027(1)
Tyrosine							0.037(1)	0.037(1)		0.031(1)				
Phenylalanine			0.035(1)	0.068(1)										
Electrophoretic mobility	Acidic	Acidic	Acidic	Neutral		Slightly acidic	Neutral	Neutral	Basic	Neutral	Slightly basic	Basic	Basic	Basic
Ninhydrin color	Orange		Yellow	Orange										
Purification method						C	C	E	E	C	E	E	E	C
Yield	10%	23%	31%	64%	81%	11%	29%	28%	20%	69%	63%	59%	14%	60%

Amino acid	C-14c	C-15a	C-15b	C-15c	C-15e	C-17a	C-17b	C-18a	C-19e	C-19f	C-20a	C-20b	C-22
Lysine	0.021(1)	0.012(1)		0.036(2)		0.071(2)	0.039(1)	0.068			0.086	0.051	0.099
Histidine		0.006(1)[a*]					0.021(1)	0.014			0.014		
Arginine			0.035(1)		0.020(1)	0.027(1)			0.013(1)	0.014		0.009	0.019
Aspartic acid						0.060(2)*	0.060(2)	0.025			0.027	0.051	0.044
Threonine	0.011(1)			0.041(2)		0.035(1)	0.035(1)	0.032			0.036		0.030
Serine				0.018(1)		0.008(0)	0.009(0)	0.017			0.007	0.033	
Glutamic acid		0.017(1)	0.038(1)			0.053(1)	0.058(2)	0.055			0.046	0.060	0.065
Proline		0.015(1)					0.026(1)	0.001			0.025		
Glycine			0.031(1)		0.040(2)*	0.036(1)	0.014(0)	0.037	0.019(2)*	0.006	0.017	0.017	0.023
Alanine	0.042(2)	0.017(1)		0.013(1)*		0.011(0)	0.013(0)	0.039	0.014(1)		0.016	0.033	0.024
Valine	*0.025(1)*		*0.030(1)*				*0.027(1)*	*0.049*			0.038		*0.036*
Methionine											0.022		0.018
Isoleucine											0.009	0.008	0.013
Leucine		0.020(1)	0.035(1)	0.026(1)	0.032(1)		0.030(1)	0.013	0.014(1)	0.043	0.020	0.011	
Tyrosine	0.016(1)[a*]					0.029(1)	0.020(1)	0.014	0.011(1)		0.022		0.016
Phenylalanine								0.020			0.012		0.017
Electrophoretic mobility	Basic	Basic	Basic	Basic	Basic	Basic		Basic	Basic	Basic	Not determined	Not determined	Basic
Ninhydrin color					Yellow				Yellow				
Purification method	C	M	M	M	M	M	M	C	C	C	E	E	E
Yield	84%	48%	57%	21%	15%	68%	31%	75%	73%	66%	34%	26%	19%

[a] These residues were confirmed by staining the peptides with Pauly reagent.

[b] Determined as methionine sulfone. No methionine was found.

Peptide T-18a: Leu–His–Lys ((Glu),[5] Pro, Ala) Thr (Leu, Ile) Lys (Residues 7 to 16)—This peptide was obtained from the tryptic digest of the intact nuclease (see Table II).

Dinitrophenyl end group: Leu or Ile.

Edman degradation (phenylthiohydantoin hydrolysis): Leu.

The amino acid composition suggested that this peptide included Peptide T-V-7b and the peptide (Leu, His) Lys. This was confirmed by tryptic digestion of Peptide T-18a, which yielded two fragments, T-18a-TI ((Leu, His) Lys) and T-18a-TII. The position of the latter fragment on a peptide map was the same as that of Peptide T-V-7b.

Trypsin Fragments:

T-18a-TI (Pauly-positive): Lys, 0.016; His, 0.017; Leu, 0.014; Ser, Glu, Gly, Ala, < 0.004. R_{FC}, 0.14; R_{FE}, 1.16.

T-18a-TII (Pauly-negative): R_{FC}, 0.70; R_{FE}, 0.55.

Carboxypeptidase B and A (10 min) (34%): (Lys), 0.010; Ile, 0.011; Leu, 0.018.

Carboxypeptidase B and A (30 min) (96%): Lys), 0.026; Ile, 0.019; Leu, 0.029; Thr, 0.017.

[5] The abbreviations used are: (Glu) and (Asp), Glu or Gln and Asp or Asn, respectively, where amide groups were not determined; DNP-, dinitrophenyl-.

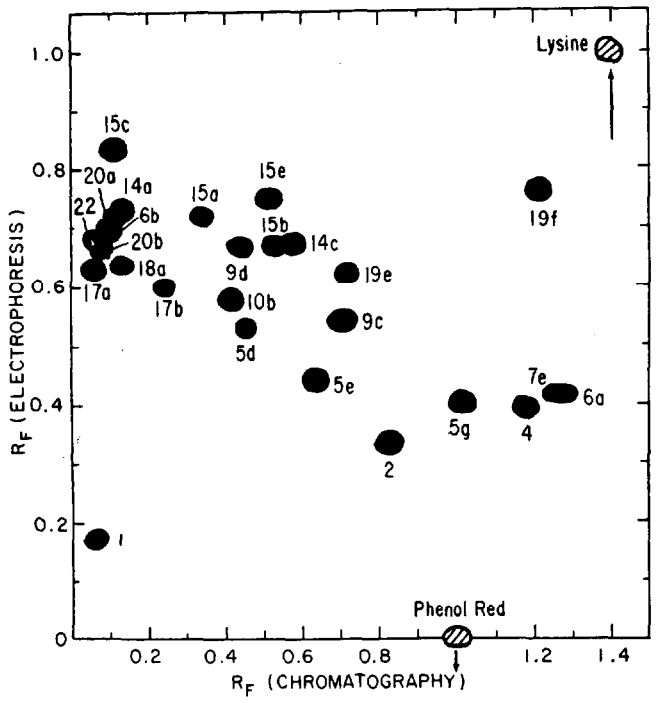

FIG. 3. Reconstructed map of the peptides obtained from the chymotryptic digest of the nuclease. The reference standards are the same as described in the text.

Fragment obtained after carboxypeptidase B and A: Lys, 0.019[6]; His, 0.026; Glu, 0.031; Pro, 0.037; Ala, 0.032; Leu, 0.014.[6] R_{FC}, 0.17; R_{FE}, 0.90.

T-V-2: Ala–Ile–Asp–Gly–Asp–Thr–Val–Lys (Residues 17 to 24)—

Dinitrophenyl end group: Ala.

Edman degradation:[7]

Stage 1 (98%): **Ala, 0.001;** Ile, 0.015(1); Asp, 0.028(2); Gly, 0.014(1); (Thr), 0.016(1); Val, 0.016(1); phenylthiohydantoin hydrolysis, Ala.

Stage 2 (100%): Ala, 0.002; **Ile, 0.002;** Asp, 0.027(2); Gly, 0.015(1); (Thr), 0.016(1); Val, 0.018(1); phenylthiohydantoin hydrolysis, Ile.

Stage 3 (86%): Ala, 0.000; Ile, 0.000; **Asp, 0.015(1);** Gly, 0.013(1); (Thr), 0.014(1); Val, 0.009(1); phenylthiohydantoin hydrolysis, Asp.

Stage 4 (107%): Ala, 0.001; Ile, 0.000; Asp, 0.017(1); **Gly, 0.008;** (Thr), 0.015(1); Val, 0.014(1).

Stage 5 (89%): Ala, 0.001; Ile, 0.001; **Asp, 0.010;** Gly, 0.006; (Thr), 0.012(1); Val, 0.014(1).

Stage 6 (90%): Ala, 0.000; Ile, 0.002; Asp, 0.008; Gly, 0.005; **Thr, 0.007;** (Val), 0.012(1).

Electrophoretic mobility: Acidic (aspartic acid residues not amidated).

Leucine aminopeptidase (19 hours): Ala, 0.043; Ile, 0.040.

T-V-1: Leu–Homoserine (Residues 25 and 26)—Dinitrophenylation gave DNP-leucine (2).

T-V-15a: Thr–Phe–Arg (Residues 33 to 35)[8]—This peptide was purified by paper electrophoresis. Dinitrophenylation gave DNP-threonine (2).

T-V-5b: Leu–Leu–Leu–Val–Asp–Thr–Pro–Gln–Thr–Lys (Residues 36 to 45)—

Dinitrophenyl end group: Leu or Ile.

Edman degradation (phenylthiohydantoin hydrolysis), five cycles: Stage 1, Leu; Stage 2, Leu; Stage 3, Leu; Stage 4, Val; Stage 5, Asp.

Electrophoretic mobility: Neutral (suggesting one amide) (see Table I, Footnote *e*).

Leucine aminopeptidase (11 hours): Leu, 0.073; Val, 0.029 (Lys, Thr, Ser, Ala, Tyr < 0.01).

Pronase digestion: Free Asp, Lys, Thr, Val, Leu.

Pronase fragment: T-V-5b-PI, R_{FC}, 0.45; R_{FE}, 0.03. Thr, 0.004; Glu, 0.005; Pro, 0.006. Pronase digestion produced free aspartic acid. Therefore, glutamic acid appears to be present as glutamine. In addition Pronase released lysine, threonine, valine, and leucine. The amount of threonine determined was approximately equal to lysine and aspartic acid, suggesting that 1 of the 2 threonine residues was not released. Accordingly, it is likely that 1 threonine residue is adjacent to the proline residue, which would be cleaved very slowly or not at all by Pronase. The Pronase digest was examined for peptide fragments by mapping, and amino acid analysis was carried out on the eluted peptides. One of these components (T-V-5b-PI) gave, qualitatively, the composition (Thr, Glu, Pro).

Acid hydrolysis of T-V-5b for 7 hours with 0.03 N HCl produced two fragments, T-V-5b-AI and T-V-5b-AII, as well as free aspartic acid. These fragments were purified by paper electrophoresis, and amino acid analyses were carried out on the eluted samples. Fragment T-V-5b-AI (R_{FE}, 0.39) appeared to give the composition (Leu_3, Val), and Fragment T-V-5b-AII (R_{FE}, 0.54) gave (Thr, Thr, Glu, Pro, Lys). Therefore it was evident that Fragments T-V-5b-AI and AII were NH_2-terminal and COOH-terminal, respectively, a conclusion consistent with the position of the aspartic acid residue determined by Edman degradation. The orange color developed by cadmium ninhydrin staining of Fragment T-V-5b-AII suggested an NH_2-terminal threonine residue. Another fragment (T-V-5b-AIII) (R_{FC}, 0.98; R_{FE}, 0.20) was obtained by longer digestion (20 hours) with 0.03 N HCl. Its amino acid composition was shown to be (Thr, Pro). Accordingly, the partial sequence Asp (Thr, Pro) Glu–Thr–Lys may be deduced, based on the significant susceptibility of the glutamic acid residue to the dilute acid hydrolysis (15). Together with the results of Pronase digestion of Peptide T-V-5b and the color produced by cadmium ninhydrin staining, the sequence of Fragment T-V-5b-AII may be deduced to be Thr–Pro–Gln–Thr–Lys. Support for the presence of threonine in the penultimate position of Peptide T-V-5b was obtained by the following experiment. Peptide T-V-5b was digested with carboxypeptidase B for 6 hours. Free lysine was released in a yield of 23% as judged by amino acid analysis of an aliquot. Hydrazinolysis was performed with the rest of the digest mixture. Free threonine was found as well as free lysine, the former in a yield of 36% based on the recovery of lysine after carboxypeptidase B. Further information on this portion of the sequence is given below (chymotryptic fragments).

F_2: His–Pro–Lys (Residues 46 to 48)—The COOH-terminal lysine residue was assigned on the basis of the specificity of trypsin. Staining of this peptide with cadmium ninhydrin did not give the yellow color characteristic of proline.

[6] Low yield was due to loss from ninhydrin staining of map.

[7] Analysis by paper electrophoresis showed the presence of 1 mole of lysine throughout the degradation.

[8] Residues 27 through 32 correspond to cyanogen bromide Fragment B (2) (see the following paper (14)).

T-V-13: Lys–Gly–Val–Glu–Lys (Residues 49 to 53)

Dinitrophenyl end group: Lys.

Edman degradation:

Stage 1 (67%): **Lys, 0.008(1);** Gly, 0.010(1); (Val), 0.009(1); Glu, 0.010(1).

Stage 2 (104%): Lys, 0.011(1); **Gly, 0.004;** (Val), 0.009(1); Glu, 0.009(1).

Stage 3 (86%): (Lys), 0.011(1); Gly, 0.004; **Val, 0.006;** Glu, 0.012(1).

Stage 4 (82%): (Lys), 0.011(1); Gly, 0.004; Val, 0.004; **Glu, 0.008.**

Leucine aminopeptidase (68%): Lys, 0.035(2); (Glu), 0.017(1); Gly, 0.016(1); Val, 0.018(1).

T-V-6: Tyr–Gly–Pro–Glu–Ala–Ser–Ala–Phe (Residues 54 to 61)—

Dinitrophenyl end group: Tyr.

Edman degradation:

Stage 1 (80%): **Tyr, 0.001;** Gly, 0.008(1); Pro, 0.010(1); Glu, 0.009(1); (Ala), 0.017(2); Ser, 0.007(1); Phe, 0.008(1); phenylthiohydantoin hydrolysis, Tyr.

Stage 2 (88%): Tyr, 0.000; **Gly, 0.002;** Pro, 0.008(1); Glu, 0.008(1); (Ala), 0.015(2); Ser, 0.006(1); Phe, 0.008(1); phenylthiohydantoin hydrolysis, Gly.

Stage 3 (102%): Tyr, 0.000; Gly, 0.002; **Pro, 0.004;** Glu, 0.009(1); (Ala), 0.015(2); Ser, 0.008(1); Phe, 0.010(1); phenylthiohydantoin hydrolysis, Pro.

Stage 4 (96%): Tyr, 0.000; Gly, 0.003; Pro, 0.003; **Glu, 0.002;** (Ala), 0.015(2); Ser, 0.009(1); Phe, 0.006(1).

Stage 5 (118%): Tyr, 0.000; Gly, 0.002; Pro, 0.000; Glu, 0.003; **Ala, 0.009(1);** Ser, 0.006(1); (Phe), 0.007(1).

Stage 6 (78%): Tyr, 0.000; Gly, 0.003; Pro, 0.003; Glu, 0.005; Ala, 0.019(1); **Ser, 0.005;** (Phe), 0.011(1).

Phenylalanine was assigned to the COOH-terminal position on the basis of the known contamination of the trypsin preparation with chymotryptic-like activity. The electrophoretic mobility indicated the presence of nonamidated glutamic acid.

T-V-10: Thr–Lys–Lys–Homoserine (Residues 62 to 65)—The amino acid composition of this peptide was previously reported (2) as (Lys_2, Thr, Glu). However, further examination, including leucine aminopeptidase digestion and Edman degradation, revealed that the residue originally identified as glutamic acid was homoserine.

Dinitrophenyl end group: Thr.

Amino acid composition: Lys, 0.028; Thr, 0.014; homoserine, 0.015; (Gly, Ser), <0.004.

Leucine aminopeptidase (69%): Lys, 0.046; Thr, 0.026; (homoserine), 0.024.

Edman degradation: Stage 1: (Lys), 0.009(2); **Thr, 0.000;** homoserine, 0.004(1).

T-11b: (Tyr, Gly, Pro, Glu, Ala) (Ser, Ala) Phe–Thr–Lys (Residues 54 to 63)—The amino acid composition of this peptide was the sum of those of Peptide T-V-6 and Thr–Lys, which appear to be fragments produced by the chymotryptic-like action of the trypsin preparation on Peptide T-11b. This assumption was supported by carboxypeptidase B and A digestions of Peptide T-11b. Free lysine, threonine, serine, alanine, and phenylalanine were released as follows.

Carboxypeptidase B (3 hours) (57%): (Lys), 0.013; Thr, 0.009; Phe, 0.011.

Carboxypeptidase B and A (16 hours) (100%): (Lys), 0.023; Thr, 0.018; Ser, 0.010; Ala, 0.022; Phe, 0.018.

Electrophoretic mobility: Neutral (glutamic acid).

T-VII-3c: Val–Glu–Asn–Ala–Lys (Residues 66 to 70)—

Dinitrophenyl end group: Val.

Electrophoretic mobility: Neutral (one free carboxyl group).

Edman degradation: The degradations of the above peptide and of T-VII-3b (Gly–Leu–Ala–Tyr; residues 88 to 91) could be carried out simultaneously on an unresolved mixture (2) of the two. These results, and analyses of a leucine aminopeptidase digest, are summarized in Table IV. The latter data show that the glutamic and aspartic acid residues are present as glutamic acid and asparagine, respectively.

TABLE IV

Sequence studies on mixture of Peptides TVII-3b and TVII-3c (see Reference 2)

Amino acid	Total composition		Edman Stage 1		Edman Stage 2		Edman Stage 3		Edman Stage 4		Leucine aminopeptidase digestion	
	3c	3b	3c	3b	3c	3b	3c	3b	3c	3b	3c	3b
	μmoles											
Valine	0.072		**0.004**		0.000		0.000		0.000		0.158	
Glutamic acid	0.089		0.031(1)		**0.008**		0.009		0.009		0.143	
Aspartic acid	0.068		0.020(1)		0.015(1)		**0.011**				0.087[a]	
Alanine	(0.105)		(1) (0.041) (1)		(1) (0.023) (1)		0.024(1)	0.000	0.014	0.000	(0.196)	
Lysine	0.079		0.029(1)		0.018(1)		0.025(1)		0.024(1)		0.116	
Glycine		0.036		**0.006**		0.000		0.000		0.000		0.069
Leucine		0.048		0.018(1)		**0.003**		0.000		0.000		0.081
Tyrosine		0.048		0.015(1)		0.008(1)		0.009(1)		**0.000**		0.086
Phenylthiohydantoin hydrolysis					Glu	Leu		Ala	Ala			
Yield			91%(Lys)		61%(Lys)		113%(Lys)		60%(Lys)		86%(Lys)	102%(Tyr)

[a] As asparagine, in the position of serine on the analyzer; identity checked by paper chromatography.

TABLE V

Sequence studies on mixture of Peptides T-VII-8a and T-VII-8b

Amino acid	Composition of total Fraction T-VII-8		Edman Stage 1		Edman Stage 2		Edman Stage 3		Edman Stage 4	Leucine aminopeptidase digestion	
	8a	8b	8a	8b	8a	8b	8a	8b	8a	8a	8b
Lysine	0.020		0.006(1)		0.006(1)		0.005(1)		0.005(1)	0.035	
Arginine		0.025		0.017(1)		0.011(1)		0.002			0.132
Aspartic acid	0.027		0.006(1)		0.006(1)		0.006(1)		0.004(1)		
Serine											0.050[a]
Glutamic acid	0.071		(2)0.023(1)[b]		0.013(2)	0.000	0.011(1)	0.000	0.007(1)	0.060[c]	
Glycine		0.036		0.002		0.000		0.000			0.065
Valine	0.018		0.006(1)		0.006(1)		0.007(1)		0.000	0.046	
Isoleucine	0.015		0.005(1)		0.000					0.030	
Phenylalanine	0.017		0.004(1)		0.005(1)		0.010(1)		0.005(1)	0.028	
Phenylthiohydantoin hydrolysis			Lys	Gly	Ile	Glu	Glu		Val		
Yield			60%(Asp)[d]		110%(Asp)[d]		89%(Asp)[d]		67%(Asp)[d]	101%(Phe)[d]	

[a] As glutamine, in the position of serine on the analyzer; identity checked by paper chromatography and with pure peptide.

[b] Assigned to both peptides.

[c] The presence of 2 moles of glutamic acid was confirmed with the purified peptide by digestion with leucine aminopeptidase.

[d] The parentheses indicate the residue on the basis of which the yields were calculated (see the text "Presentation of Amino Acid Sequence").

T-VII-8a: Lys–Ile–Glu–Val (Glu, Phe) Asn–Lys (Residues 71 to 78)—

Dinitrophenyl end group: Lys.

Electrophoretic mobility: Neutral (two nonamidated carboxyl groups).

Edman degradation: Carried out on a mixture (2) of T-VII-8a and Peptide T-VII-8b (Gly–Gln–Arg; residues 79 to 81). These results are summarized in Table V.

Leucine aminopeptidase: Also summarized in Table V. Two moles of glutamic acid were released together with 1 mole of isoleucine, valine, and phenylalanine. Only 1 mole of lysine appeared to be present in the digest. Neither aspartic acid nor asparagine was found. An undigested fragment, Asn–Lys, presumably remained. Data to be presented below support the presence of a penultimate asparaginyl residue.

T-VII-8b: Gly–Gln–Arg (Residues 79 to 81)—

Dinitrophenyl end group: Gly; cadmium ninhydrin staining yielded a yellow color.

Edman degradation: See Table V.

Electrophoretic mobility: Basic.

T-VII-2c: Thr–Asp–Lys (Residues 82 to 84)—

Dinitrophenyl end group: Thr; orange color with cadmium ninhydrin.

Leucine aminopeptidase: Thr (96%).

Electrophoretic mobility: Neutral.

T-VII-11a: Tyr–Gly–Arg (Residues 85 to 87)—

Dinitrophenyl end group: Tyr.

Further information on this portion of the sequence is given below in connection with the chymotryptic fragment, C-15e.

T-VII-10a:[9] *Thr–(Asp)–(Lys, Tyr, Gly)–Arg (Residues 82 to 87)*

Dinitrophenyl end group: Thr (confirmed by Edman degradation and leucine aminopeptidase digestion).

Acid hydrolysis (0.03 N HCl): Three components on the peptide map. Elution, hydrolysis, and analysis yielded (*a*) Thr, 0.018; Asp, Gly < 0.004; (*b*) Asp; (*c*) Lys, 0.019[10]; Arg, 0.045; Gly, 0.058; Tyr, 0.025[10] (R_{FC}, 0.27; R_{FE}, 0.98).

T-VII-3b: Gly–Leu–Ala–Tyr (Residues 88 to 91)—

Dinitrophenyl end group: Gly; yellow color with cadmium ninhydrin.

Edman degradation: See Table IV.

T-VII-5a: Ile–Tyr–Ala–Asp–Gly–Lys (Residues 92 to 97)—

Dinitrophenyl end group: Leu or Ile.

Edman degradation[11]:

Stage 1 (69%): **Ile, 0.001;** Tyr, 0.021(1); Ala, 0.025(1); (Asp), 0.018(1); Gly, 0.029(1); phenylthiohydantoin hydrolysis, Ile.

Stage 2 (115%): Ile, 0.001; **Tyr, 0.002;** Ala, 0.018(1); (Asp), 0.019(1); Gly, 0.017(1); phenylthiohydantoin hydrolysis, Tyr.

Stage 3 (81%): Ile, 0.001; Tyr, 0.002; **Ala, 0.005;** (Asp), 0.017; Gly, 0.024(1); phenylthiohydantoin hydrolysis, Ala.

Stage 4 (83%): Ile, 0.000; Tyr, 0.001; Ala, 0.003; **Asp, 0.006;** (Gly), 0.020(1).

Leucine aminopeptidase (19 hours) (107%): (Ala), 0.061; Ile, 0.049; Tyr, 0.080.

Electrophoretic mobility: neutral.

F_{24}: *(Gly, Leu, Ala) Tyr (Ile, Tyr, Ala, Asp, Gly) Lys (Residues 88 to 97)*—The amino acid composition of this peptide was equal to the sum of the compositions of Peptides T-VII-3b and T-VII-5a. The latter were presumably formed by the chymotryptic-like activity in the trypsin preparation.

[9] The peptide map of Fraction T-VII-10 (2) showed a single Pauly-positive spot (2). However, the amino acid analysis of Peptide T-VII-10a (ion exchange chromatographic yield, 26%) was only qualitative, because of contamination of Fraction T-VII-10 with minor components.

[10] Low yield was due to ninhydrin staining and destruction during hydrolysis.

[11] The basic amino acids were not determined on the analyzer. Analyses by paper electrophoresis showed 1 eq of lysine throughout.

T-III-6: Val–Asn–Glu–Ala–Leu–Val–Arg (Residues 99 to 105)[12]—

Dinitrophenyl end group: Val.

Edman degradation[13]:

Stage 1 (104%): **Val, 0.036(1);** Asp, 0.018(1); Glu, 0.025(1); Ala, 0.029(1); (Leu), 0.025(1); phenylthiohydantoin hydrolysis, Val.

Stage 2 (89%): Val, 0.019(1); **Asp, 0.005;** Glu, 0.020(1); Ala, 0.020(1); (Leu), 0.021(1); phenylthiohydantoin hydrolysis, Asp.

Stage 3 (89%): Val, 0.022(1); Asp, 0.002; **Glu, 0.006;** (Ala), 0.020(1); Leu, not determined; phenylthiohydantoin hydrolysis, Glu.

Stage 4 (96%): (Val), 0.021(1); Asp, 0.004; Glu, 0.008; **Ala, 0.010;** Leu, 0.014(1).

Leucine aminopeptidase[14]: Asn, 0.054; Glu, 0.065; Ala, 0.066; Val, 0.208; Leu, 0.064.

Carboxypeptidase B (30 min): Arg, 0.063; Val, 0.013.

Carboxypeptidase B (30 min) followed by carboxypeptidase A (20 min): Arg, 0.066; Val, 0.075; Leu, 0.082.

T-III-7b: Gln–Gly–Leu–Ala–Lys (Residues 106 to 110)—

Dinitrophenyl end group: Glu.

Edman degradation:

Stage 1 (86%) **Glu, 0.002;** Gly, 0.005(1); Leu, 0.007(1); Ala, 0.006(1); (Lys), 0.006(1); phenylthiohydantoin hydrolysis, Glu.

Leucine aminopeptidase (10 hours) (60%): Gln, 0.015; Gly, 0.014; (Ala), 0.018; Leu, 0.016.

Carboxypeptidase B (3 hours) (66%): (Lys), 0.018; Ala, 0.005.

Carboxypeptidase B and A (5 hours) (103%): (Lys), 0.031; Ala, 0.032; Leu, 0.026; Gly, 0.008; Gln, 0.008.

Since glutamine was known to be the NH_2 terminus, carboxypeptidase digestion provided the above sequence. The basic amino acids in the leucine aminopeptidase hydrolysate were not determined on the analyzer, but lysine was found on paper chromatography. Glutamine, which appeared as serine on the analyzer, was identified by paper chromatography.

T-III-4c: Val–Ala–Tyr (Residues 111 to 113)—This peptide, upon dinitrophenylation, gave DNP-valine. The carboxyl-terminal tyrosine residue is consistent with the chymotryptic-like activity of the trypsin preparation.

T-III-12a: Val–Tyr–Lys–Pro–Asn–Asn–Thr–His–Glu–Gln–Leu–Leu–Arg (Residues 114 to 126)—Fraction T-III-12 gave a single ninhydrin-positive spot on the peptide map. Dinitrophenylation of this component gave exclusively valine as the dinitrophenyl end group (2). Leucine aminopeptidase released valine and tyrosine and a smaller, but significant, amount of alanine. (Alanine was found in the hydrolysate of Fraction T-III-12, but was not assigned to Peptide T-III-12 in the previous report (2).) Pronase digestion also yielded alanine in the same relative amount as did leucine aminopeptidase digestion. Peptide T-20 (see Table II) had the same amino acid composition as Fraction T-III-12 except that alanine was missing. Free valine and tyrosine were again produced by leucine aminopeptidase digestion.

Peptide T-III-4c, Val–Ala–Tyr (residues 111 to 113; see above), was found in a relatively low yield (16%), compared with other peptides (30 to 70%) in the tryptic digest of cyanogen bromide Fragment E (see Table II). The same peptide was found (as T-5b and F_{27}) in the tryptic digest of the nuclease (see Tables I and II). The presence of alanine in Fraction T-III-12 was therefore interpreted as follows. Fraction T-III-12 contained two peptides, T-III-12a and T-III-12b, which had occupied closely neighboring positions on the peptide map. Peptide T-III-12a had the NH_2-terminal sequence Val–Tyr–. Peptide T-III-12b, with the NH_2-terminal sequence Val–Ala–Tyr–Val–Tyr–, overlaps Peptides T-III-4c and T-III-12a. These conclusions were supported by the following experiment. Fraction T-III-12 was incubated with trypsin for 22 hours and peptide maps were prepared. A new Pauly-positive spot was found at the position on the map corresponding to that of Peptide T-III-4c.

The further study of the sequence of Peptide T-III-12a was made on both Fraction T-III-12 and Peptide T-20. Carboxypeptidase B released only arginine. Further incubation with carboxypeptidase A released 2 moles of leucine. Thus the COOH-terminal sequence was interpreted to be –Leu–Leu–Arg. Dilute acid hydrolysis with 0.03 N HCl produced free aspartic acid in a yield of 83% and two fragments, T-20-AI and T-20-AII (see Table VI), which were purified by two-dimensional mapping. The amino acid compositions of these fragments were (Val, Tyr, Leu, Pro) and (His, Arg, Thr, Glu, Glu, Leu, Leu), respectively. Fragment T-20-AI is evidently the NH_2-terminal portion of Peptide T-III-12a. Since leucine aminopeptidase digestion of Peptide T-III-12a released valine and tyrosine residues as described above, the proline residue should not be next to valine and tyrosine. Thus, the sequence of Fragment T-20-AI was deduced as Val–Tyr–Lys–Pro. Fragment T-20-AII was subjected to timed digestion with leucine aminopeptidase to determine the NH_2-terminal sequence. Threonine was released first, followed by histidine. This is consistent with the orange color developed by staining Fragment T-20-AII with cadmium ninhydrin. From the above results, the sequence Val–Tyr–Lys–Pro–(Asp)–(Asp)–Thr–His–(Glu)–(Glu)–Leu–Leu–Arg was deduced for Peptide T-III-12a.

Since the electrophoretic mobility of Peptide T-III-12a was basic at pH 6.5, at least two carboxyl groups of four should be amidated. The digestion of Peptide T-III-12a with Pronase released a very small amount of histidine. Neither glutamic acid nor proline was found. However, valine, tryrosine, lysine, asparagine, threonine, glutamine, and leucine were released. Glutamine and asparagine were identified by ascending paper chromatography with 80% aqueous pyridine and subsequent paper electrophoresis at pH 6.5. Since Pronase did not cleave the peptide bond involving the carboxyl group of proline, the released asparagine residue must be adjacent to threonine. The mixed digestion of Peptide T-III-12a with Pronase and leucine aminopeptidase released equimolar amounts of glutamic acid and histidine in addition to all other residues released by Pronase alone. However, proline was not found. These results indicated that the fragment His–Glu remained almost intact during Pronase digestion, as well as Pro–Asp (or Pro–Asn). Since no acidic

[12] Residue 98 was deduced to be methionine (14). Free homoserine was found in the tryptic digest of cyanogen bromide fragment D by Peptide mapping and amino acid analysis.

[13] The basic amino acids were determined by paper electrophoresis. Approximately 1 eq of arginine was present through all stages.

[14] The basic amino acids were not determined. Asparagine, which appeared as serine on the amino acid analyzer, was identified by paper chromatography.

TABLE VI

Sequence studies on Peptide T-III-12a

1. Leucine aminopeptidase digestion,[a] 19 hrs; 86%	Ala, 0.013;[b] (Val), 0.093; Tyr, 0.051
2. 1 + carboxypeptidase B, 80 min; 130%	Ala, 0.009;[b] (Val), 0.040; Tyr, 0.027; Arg, 0.025
3. 2 + carboxypeptidase A, 6 hrs; 110%	Ala, 0.007;[b] (Val), 0.032; Tyr, 0.031; Arg, 0.019; Leu, 0.055
4. Pronase, 20 hrs; 52%	Lys, 0.047; His, 0.005; Arg, 0.092; Thr, 0.047; Ser,[c] 0.041; Ala,[b] 0.019; (Val), 0.097; Met <0.003; Leu, 0.104; Tyr, 0.094
Acid fragments[d]	
T-20-AI R_{FC}, 0.45; R_{FE}, 0.73)	Lys, 0.031(1); Pro, 0.017(1); Gly, 0.005(0); Val, 0.031(1): Leu, 0.003(0); Tyr, 0.042(1)
T-20-AII R_{FC}, 0.47; R_{FE}, 0.61)	Lys, 0.015(0); His, 0.041(1); Arg, 0.049(1); Asp, 0.005(0); Thr, 0.022(1); Glu, 0.091(2); Gly, 0.005(0); Val, 0.012(0); Leu, 0.100(2); Tyr, 0.018(0)

[a] Basic amino acids were not determined.

[b] See the text.

[c] The peak at the serine position was supposedly due to asparagine, glutamine, or both (see the text).

[d] Peptide T-20 was used for dilute acid hydrolysis (see Table I).

components were found upon electrophoresis of the Pronase digest at pH 6.5, the latter fragment must contain asparagine. Thus the distribution of amide groups in Peptide T-III-12a is as shown above. Pertinent results are summarized in Table VI.

T-III-9c: Lys–Glu–Lys (Residues 127 to 129)—

Dinitrophenyl end group: Lys.

Edman degradation:

Stage 1 (80%): **Lys, 0.01;** (Glu), 0.007.

Leucine aminopeptidase (19 hours) (100%): Lys, 0.069; Glu, 0.035.

T-III-5b: Glu–Lys (Residues 128 and 129)—

Dinitrophenyl end group: Glu.

Leucine aminopeptidase: Lys, 0.026; Glu, 0.027.

T-III-8: Lys–Ser–Glu–Ala–Gln–Ala–Lys[15] *(Residues 130 to 136)*—

Dinitrophenyl end group: Lys.

Electrophoretic mobility: Basic (amidation of at least 1 glutamic acid residue).

Edman degradation:

Stage 1 (85%): **Lys, 0.016(1);** Ser, 0.012(1); Glu, 0.023(2); (Ala), 0.023(2).

Stage 2 (68%): (Lys), 0.011(1); **Ser, 0.007;** Glu, 0.036(2); Ala, 0.029(2).

Stage 3 (73%): (Lys), 0.014(1); Ser, 0.002; **Glu, 0.019(1);** Ala, 0.028(2).

[15] Chromatographic Fraction T-11, containing four peptides—T-11a, Lys–Ile–Glu–Val (Glu, Phe) Asn–Lys; T-11b, (Tyr, Gly, Pro, Glu, Ala) (Ser, Ala) Phe–Thr–Lys; T-11c, Lys–Ser–Glu–Ala–Gln–Ala–Lys; and T-11d, Gly–Gln–Arg—was digested with carboxypeptidase B for 3 hours. COOH-terminal residues, lysine and arginine, were liberated in yields of 66 and 68%, respectively. Hydrazinolysis of this digest yielded only threonine (0.024) and alanine (0.026), in total yields of 28 and 21%, respectively, consistent with the penultimate residues shown above.

Leucine aminopeptidase (19 hours) (112%): Lys, 0.113; Ser,[16] 0.081; Glu, 0.063; (Ala), 0.130.

Carboxypeptidase B (3 hours) (56%): (Lys), 0.026; Ala, 0.005.

Carboxypeptidase B and A (2 hours) (28%): Lys, 0.031; (Ala), 0.016.

Carboxypeptidase B and A (17 hours) (73%): Lys, 0.049; Gln, 0.044; (Ala), 0.058.

T-III-2: Leu–Asn–Ile–Trp–Ser–Glu–(Asp)–Asp–(Ala, (Asp))–Ser–Gly–Gln (Residues 137 to 149)—

Dinitrophenyl end group: Leu or Ile.

Carboxypeptidase A: Gln.

Carboxypeptidase followed by hydrazinolysis: Gly (30% yield).

Subtilisin cleaved Peptide T-III-2 completely to produce two fragments, T-III-2-SI (Asp, Ile, Leu, Trp) (R_{FC}, 1.48; R_{FE}, 0.37) and T-III-2-SII (Asp_3, Ser_2, Glu_2, Gly, Ala) (R_{FC}, 0.00; R_{FE}, 0.007) (2). The electrophoretic mobilities of Fragments T-III-2-SI and T-III-2-SII were neutral and acidic, respectively. Therefore, the aspartic acid residue of Fragment T-III-2-SI may be deduced to be in the amidated form.

Leucine aminopeptidase released 1 mole each of free glutamic acid and aspartic acid from the mixture of the two fragments, in addition to leucine, isoleucine, tryptophan, and serine (the last peak on the analyzer presumably including asparagine). Since the two subtilisin fragments contained different amino acid residues with the exception of asparagine, Edman degradation was performed through several stages with the mixture of the two fragments.

The results of the first three stages were as follows (final details of the elucidation of the total sequence of Peptide T-III-2 will be presented in the subsequent, and final, paper on the nuclease sequence (14)).

Edman degradation:

Stage 1 (89%): Fragment T-III-2-SI: **Leu, 0.002;** Asp, 0.013(1)[17]; Ile, 0.011(1); phenylthiohydantoin hydrolysis, Leu. Fragment T-III-2-SII: **Ser, 0.012(1);** Glu, 0.031(2); Asp, 0.037(3)[17]; Ala, 0.015(1); (Gly), 0.015(1).

Stage 2 (100%): Fragment T-III-2-SI: Leu, 0.003; **Asp, 0.000**[17]; Ile, 0.008(1). Fragment T-III-2-SII: Ser, 0.011(1); **Glu, 0.019(1);** Asp, 0.040(3);[17] Ala, 0.014(1); (Gly), 0.013(1).

Stage 3 (104%): Fragment T-III-2-SI: Leu, 0.002; Asp, 0.000; **Ile, 0.002;** phenylthiohydantoin hydrolysis, Ile. Fragment T-III-2-SII: Ser, 0.015(1); Glu, 0.018(1); **Asp, 0.032(2);** Ala, 0.016; (Gly), 0.016(1).

Pronase digestion of Peptide T-2 (see Tables I and II) produced several peptide fragments together with leucine, asparagine, isoleucine, and tryptophan. Peptide fragments were purified by two-dimensional mapping. One fragment, T-2-P-a, contained 2 eq of aspartic acid and 1 eq of alanine and released 0.5 mole of aspartic acid upon leucine aminopeptidase digestion (16 hours) without liberation of any other amino acids. Another fragment, T-2-P-b, contained serine, glutamic acid, and aspartic acid in addition to the amino acid residues of Fragment T-2-P-a. The amino acid compositions of these Pronase fragments are as follows.

T-2-P-a (R_{FC}, 0.22; R_{FE}, 0.09): Asp, 0.057(2); Ala, 0.025(1); Ser, Glu, Gly < 0.008.

[16] The peak at the serine position included glutamine, which was identified by paper chromatography.

[17] The assignment of values for aspartic acid was based on the amino acid composition of the two fragments.

T-2-P-b (R_{FC}, 0.23; R_{FE}, 0.05): Asp, 0.084(3); Ser, 0.016(1); Glu, 0.029(1); Ala, 0.028(1); Gly < 0.006.

T-2-P-c (R_{FC}, 0.29; R_{FE}, 0.34) Ser, 0.025(1); Glu, 0.060(1); Gly, 0.049(1); Asp < 0.006.

A summary of the amino acid sequences of the tryptic peptides is given in Table VII.

Partial Amino Acid Sequences of Chymotryptic Peptides

Complete or nearly complete sequences were determined for essentially all the chymotryptic peptides indicated in Fig. 2 and Table III. However, the mass of experimental description has been reduced for presentation here to a point sufficient for the establishment of the overlaps that permit the assembly of tryptic fragments in the correct order, and for the completion of small portions of sequence in these latter fragments that had not been unequivocally established.

C-1: Ser–Glu–Asn (Asp, Ala) Asp–Ser (Gln, Gly)—

Cadmium ninhydrin color: Orange (Ser).

Leucine aminopeptidase (5 hours) (32%): Ser, 0.010; Glu, 0.011; Asp, 0.005; (Ala), 0.003.

TABLE VII

Summary of amino acid sequences of tryptic peptides

Cyanogen bromide fragment	Peptide	Sequence
A	T-V-8a	Ala–Thr–Ser–Thr–Lys
A	T-V-7b	Glu–Pro–Ala–Thr (Leu, Ile) Lys
A	T-18a	Leu–His–Lys ((Glu), Pro, Ala) Thr (Leu, Ile) Lys
A	T-V-2	Ala–Ile–Asp–Gly–Asp–Thr–Val–Lys
A	T-V-1	Leu–homoserine
C	T-V-15a	Thr–Phe–Arg
C	T-V-5b	Leu–Leu–Leu–Val–Asp–Thr–Pro–Gln[a]–Thr–Lys
C	F_2	His–Pro–Lys
C	T-V-13	Lys–Gly–Val–Glu–Lys
C	T-V-6	Tyr–Gly–Pro–Glu–Ala–Ser–Ala–Phe
C	T-V-10	Thr–Lys–Lys–homoserine
C	T-11b	(Tyr, Gly, Pro, Glu, Ala) (Ser, Ala) Phe–Thr–Lys
D	T-VII-3c	Val–Glu–Asn–Ala–Lys
D	T-VII-8a	Lys–Ile–Glu–Val (Glu, Phe) Asn–Lys
D	T-VII-8b	Gly–Gln–Arg
D	T-VII-2c	Thr–Asp–Lys
D	T-VII-11a	Tyr–Gly–Arg
D	T-VII-10a	Thr–(Asp) (Lys, Tyr, Gly) Arg
D	T-VII-3b	Gly–Leu–Ala–Tyr
D	T-VII-5a	Ile–Tyr–Ala–Asp–Gly–Lys
D	F_{24}	(Gly, Leu, Ala) Tyr (Ile, Tyr, Ala, Asp, Gly) Lys
E	T-III-6	Val–Asn–Glu–Ala–Leu–Val–Arg
E	T-III-7b	Gln–Gly–Leu–Ala–Lys
E	T-III-4c	Val–Ala–Tyr
E	T-III-12a	Val–Tyr–Lys–Pro–Asn[a]–Asn–Thr–His–Glu–Gln–Leu–Leu–Arg
E	T-III-9c	Lys–Glu–Lys
E	T-III-5b	Glu–Lys
E	T-III-8	Lys–Ser–Glu–Ala–Gln–Ala–Lys
E	T-III-2	Leu–Asn–Ile–Trp–Ser–Glu–(Asp)–Asp (Ala, (Asp)) Ser–Gly–Gln

[a] The assignment of amide groups is tentative (see the text).

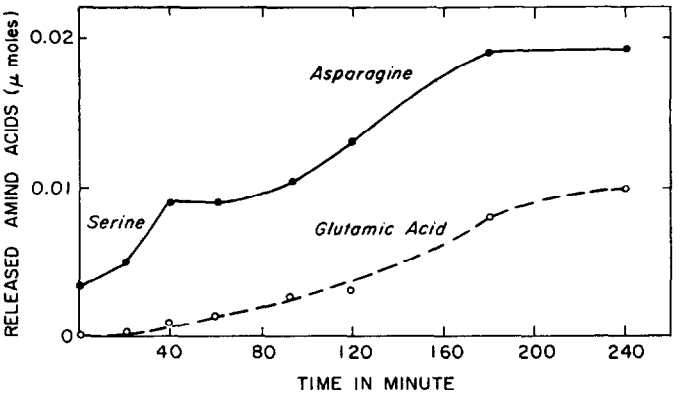

FIG. 4. Timed digestion of Peptide C-1 with leucine aminopeptidase. The reaction mixture, in 0.21 ml of 0.05 M NH_4HCO_3, pH 8.0, and 0.001 M $MgCl_2$, contained 0.15 μmole of Peptide C-1 and 25 μg of leucine aminopeptidase. Incubation was conducted at 37°. Aliquots of 20 μl were taken at the indicated times, freeze-dried, and analyzed with an amino acid analyzer. Only two peaks, at the positions of serine and glutamic acid, were observed with all samples. Calculations involving the peak at the serine position were made by assuming that serine was released first and that the release of asparagine produced the subsequent increment in the peak (see the text). Theoretical yield was 0.0125 μmole for each amino acid.

Leucine aminopeptidase (19 hours) (83%): Asp, 0.018; Ser, 0.023; Glu, 0.010; Gly, 0.006; (Ala), 0.010.

The serine peak contained asparagine and glutamine, identified by paper chromatography.

Leucine aminopeptidase digestion converted nearly all of C-1 to free amino acids, including 2 eq of aspartic acid and 1 eq of glutamic acid. These analyses indicate the amidation of 1 residue of glutamic acid. Aliquots taken early during timed hydrolysis with leucine aminopeptidase contained 1 eq of serine and only traces of glutamic acid. The liberation of asparagine followed the release of these amino acids (Fig. 4).

Dilute acid hydrolysis of C-1 gave two peptide fragments, purified by peptide mapping (C-1-A-I and C-1-A-II), in addition to free aspartic acid and alanine.

C-1-AI (R_{FC}, 0.32; R_{FE}, 0.28): Ser, 0.022; Glu, 0.54.

C-1-A-II (R_{FC}, 0.43; R_{FE}, 0.28): Ser, 0.053; Glu, 0.081; Gly, 0.077; Asp and Ala < 0.020.

The values for serine are presumably low because of destruction by ninhydrin and acid hydrolysis.

Edman degradation of C-1-AII:

Stage 1 (56%): **Ser, 0.005;** (Glu), 0.023(1); Gly, 0.020(1); Asp, 0.005(0).

These results indicate that Fragments C-1-AI and C-1-AII are placed at the NH_2- and COOH-terminal ends of Peptide C-1, respectively.

C-2: Gly–Pro–Glu–Ala–Ser–Ala–Phe—

Cadmium ninhydrin color: Yellow (Gly or Pro). The latter is excluded on the basis of the unsusceptibility of prolyl bonds to chymotrypsin.

Carboxypeptidase A (10 min): Ala, 0.021; Phe, 0.049.

Carboxypeptidase A (20 min): Ala, 0.030; Phe, 0.046.

Carboxypeptidase A (60 min): Ala, 0.033; Phe, 0.041; Ser, 0.005.

Carboxypeptidase A (120 min): Ala, 0.037; Phe, 0.041; Ser, 0.007.

The progressive increase in alanine following the appearance of serine is consistent with the sequence shown above.

The electrophoretic mobility of this peptide was acidic, indicating a nonamidated glutamic acid residue.

C-4: Thr–Phe—This peptide gave an orange color upon cadmium ninhydrin staining, indicating NH_2-terminal threonine. A COOH-terminal phenylalanine residue is consistent with the specificity of chymotrypsin.

C-5d: (Lys, Asp, Gly, Ala) Met—Electrophoretic neutrality indicated aspartic acid rather than asparagine. A COOH-terminal methionine residue was assigned on the basis of the specificity of chymotrypsin.

C-5e: ((Asp), Ala, Gly) Lys ((Asp), (Glu), Ala, Val, Met, Leu)—Tryptic digestion of this peptide produced two fragments, which were purified by paper electrophoresis. The amino acid compositions of these fragments accounted for that of the parent peptide, C-5e, as follows.

Peptide C-5e-TI (R_{FE}, 0.58): Lys, 0.006(1); Asp, 0.007(1); Gly, 0.008(1); Ala, 0.005(1).

Fragment C-5e-TII (R_{FE}, 0.39): Asp, 0.009(1); Glu, 0.007(1); Ala, 0.008(1); Val, 0.005(1); Met, 0.006(1); Leu, 0.007(1).

Fragment C-5e-TII, which contained no lysine, was assigned as the COOH-terminal portion of Peptide C-5e.

C-9c: Ile–Lys (Ala, Ile) (Asp, Gly, Asp, Thr) Val–Lys–Leu—One stage of Edman degradation indicated NH_2-terminal isoleucine.

Stage 1 (47%): **Ile, 0.007(1);** Lys, 0.010(2); Ala, 0.007(1); Asp, 0.016(2); Gly, 0.009(1); Thr, 0.008(1); Val, 0.005(1); (Leu), 0.007(1).

One tryptic fragment derived from Peptide C-9c (purified by paper chromatography; R_{FC}, 0.76) had the same amino acid composition, lacking 1 residue each of isoleucine and lysine: Lys, 0.015(1); Asp, 0.031(2); Thr, 0.015(1); Gly, 0.022(1); Ala, 0.014(1); Val, 0.018(1); Ile, 0.016(1); Leu, 0.018(1).

Carboxypeptidase A treatment of peptide C-9c released only leucine, and the subsequent addition of carboxypeptidase B to the incubation mixture resulted in the additional liberation of lysine and valine, indicating the COOH-terminal sequence to be –Val–Lys–Leu. Leucine aminopeptidase released 1 mole each of lysine and alanine and 2 moles of isoleucine. Accordingly, the NH_2-terminal sequence was deduced to be Ile–Lys (Ala, Ile). Peptide C-9c was electrophoretically neutral, indicating that neither aspartic acid residue was amidated.

C-9d: (Lys, Gly, Pro, Gln) Met—Digestion with carboxypeptidase A and B released only methionine. Upon electrophoresis, the peptide was basic, indicating amidation of the glutamic acid residue.

C-14a: Lys–Pro–Asn (Asn, Thr, His, Gln, Glu) Leu—Carboxypeptidase A released only leucine. Edman degradation indicated the NH_2-terminal sequence to be Lys–Pro–.

Edman degradation:

Stage 1 (82%): **Lys, 0.000;** Pro, 0.012(1); Asp, 0.023(2); Thr, 0.011(1); His, 0.009(1); Glu, 0.020(2); (Leu), 0.012(1).

Stage 2 (75%): Lys, 0.000; **Pro, 0.000;** Asp, 0.015(2); Thr, 0.007(1); His, 0.007(1); Glu, 0.016(2); (Leu), 0.009(1).

Hydrolysis with 0.03 N HCl produced free aspartic acid and two fragments, C-14a-AI and C-14a-AII, purified by two-dimensional peptide mapping. The amino acid analysis of the purified fragments indicated the compositions (Lys, Pro) and (His, Asp, Thr, Glu_2, Leu), respectively.

Fragment C-14a-AI (R_{FC}, 0.73; R_{FE}, 0.94): Lys, 0.017(1); Pro, 0.048(1); Gly < 0.005.

Fragment C-14a-AII (R_{FC}, 0.5; R_{FE}, 0.29): His, 0.027(1); Asp, 0.031(1): Thr, 0.025(1); Glu, 0.054(2); Leu, 0.031(1); Gly < 0.008.

The low value for lysine in C-14a-AI is presumably due to destruction by ninhydrin. These analyses, together with the results of Edman degradation and carboxypeptidase A digestion, indicate that Fragments C-14a-AI and C-14a-AII are NH_2-terminal and COOH-terminal parts of Peptide C-14a, respectively.

The electrophoretic mobility of Peptide C-14a was basic, indicating the amidation of at least 3 of the 4 residues of aspartic and glutamic acids. The peptide was subjected to leucine aminopeptidase digestion, after removal of 2 NH_2-terminal residues by two cycles of Edman degradation as described above. Digestion proceeded through the entire peptide. No aspartic acid was released, but glutamic acid and asparagine or glutamine (or both) were released, the latter two appearing in the serine position in the amino acid analyzer effluent.

C-14c: Ala–Lys (Ala, Val) Tyr—Incubation with carboxypeptidase A for 5 hours released 1 mole each of alanine, valine, and tyrosine (80% recovery); Ala, 0.015; Val, 0.014; Tyr, 0.017. These results suggest that the fourth position from the COOH terminus is occupied by a lysine residue. Tyrosine was assigned to the COOH-terminal position on the basis of the specificity of chymotrypsin.

C-15a: His–Lys ((Glu), Pro, Ala, Thr) Leu—Two stages of Edman degradation indicated the NH_2-terminal sequence to be His–Lys–.

Stage 1 (116%): **His, 0.002;** Lys, 0.003(1); Glu, 0.005(1); Pro, 0.005(1); Ala, 0.005(1); Thr, 0.006(1); (Leu), 0.006(1).

Stage 2 (83%); His, 0.002; **Lys, 0.001;** Glu, 0.008(1); Pro, 0.005(1); Ala, 0.008(1); Thr, 0.007(1); (Leu), 0.010.

Leucine aminopeptidase digestion for 16 hours failed to release free amino acids. Carboxyl-terminal leucine was assigned on the basis of the specificity of chymotrypsin. Pronase released free leucine (together with small amounts of threonine).

C-15b: (Val, Arg) (Gln, Gly) Leu—The COOH-terminal residue was assigned on the basis of the specificity of chymotrypsin. Peptide C-15b was basic on electrophoresis, indicating amidation of the glutamic acid residue. Carboxypeptidase A digestion (17 hours) released leucine (111%) together with small amounts of glycine and glutamine (on the position of serine on the amino acid analyzer).

C-15c: Ala (Lys, Thr_2, Ser) Lys–Leu—Edman degradation gave NH_2-terminal alanine.

Stage 1 (90%): **Ala, 0.004;** Lys, 0.007(1); Thr, 0.014(2); Ser, 0.008(1); Leu, 0.010(1).

The low yield of lysine (70%) was presumably due to partial destruction by the Edman reaction. Successive digestion with carboxypeptidase A and B showed the COOH-terminal sequence to be –Lys–Leu.

Carboxypeptidase A (3 hours) (92%): Leu, 0.012.

Carboxypeptidase A and B (7 hours) (85%): Lys, 0.011; (Leu), 0.011.

C-15e: Gly–Arg–Gly–Leu—An NH_2-terminal glycine residue was indicated by the yellow color produced by cadmium ninhydrin staining. Carboxypeptidase A released free leucine in a

yield of 109% after 17 hours of digestion, together with small amounts of glycine.

C-17a: (Asp)–Lys (Gly, (Glu)) Arg (Thr, (Asp), Lys) Tyr—Trypsin cleaved this peptide into two fragments.

C-17a-TI (R_{FC}, 0.06; R_{FE}, 0.66): Lys, 0.026(1); Arg, 0.039(1); Asp, 0.022(1); Glu, 0.030(1); Gly, 0.028(1).

C-17a-TII (R_{FC}, 0.41; R_{FE}, 0.51): Lys, 0.022(1); Asp, 0.026(1); Thr, 0.019(1); Tyr, 0.022(1); Glu, Gly < 0.004.

Fragment C-17a-TII, containing tyrosine, was assigned to the COOH-terminal portion of C-17a.

The NH_2-terminal sequence was determined, by two cycles of Edman degradation, to be (Asp)–Lys–.

Stage 1 (90%): **Asp, 0.011(1);** Lys, 0.016(2); Gly, 0.011(1); Glu, 0.014(1); Arg, 0.007(1); (Thr), 0.009(1); Tyr, 0.003(1); Ala < 0.005.

Stage 2 (80%): Asp, 0.010(1); **Lys, 0.007(1);** Gly, 0.008(1); Glu, 0.012(1); Arg, 0.009(1); (Thr), 0.008(1); Tyr, 0.003(1).

Some destruction of tyrosine during the purification of Peptide C-17a was observed earlier (see Table III). Amide groups were not determined.

C-18a: Leu–Val ((Asp), Thr, Pro, (Glu), Thr) Lys (His, Pro, Lys) (Lys, Gly, Val, (Glu)) Lys (Tyr, Gly, Pro, (Glu), Ala_2, Ser, Phe)—Tryptic digestion of C-18a yielded four major fargments: C-18a-TI, (Leu, Val, (Asp), Thr_2, Glu, Pro, Lys); C-18a-TII, (His, Pro, Lys); C-18a-TIII, (Glu, Gly, Val, Lys_2); and C-18a-TIV, (Ser, Glu, Pro, Gly, Ala_2, Tyr, Phe). Two larger, overlapping fragments, C-18a-TV and C-18a-TVI, were also produced. Fragments were purified by two-dimensional peptide mapping. The amino acid ecmpositions of the purified peptides

TABLE VIII

Amino acid compositions of peptide fragments produced by tryptic digestion of chymotryptic Peptide C-18a

Tryptic fragment	Amino acid composition
C-18a-TI (R_{FC}, 0.42; R_{FE}, 0.43)	Lys, 0.019(1); Asp, 0.020(1); Thr, 0.031(1-2); Glu, 0.019(1); Pro, 0.021(1); Val, 0.029(1); Leu, 0.027(1)
C-18a-TII (R_{FC}, 0.10; R_{FE}, 0.96)	Lys, 0.033(1-2); His, 0.016(1); Pro, 0.013(1)
C-18a-TIII (R_{FC}, 0.09; R_{FE}, 0.78)	Lys, 0.033(2); Glu, 0.019(1); Gly, 0.018(1); Val, 0.023(1)
C-18a-TIV (R_{FC}, 0.99; R_{FE}, 0.27)	Ser, 0.022(1); Glu, 0.029(1); Pro, 0.031(1); Gly, 0.028(1); Ala, 0.055(2); Tyr, 0.017(1); Phe, 0.032(1)
C-18a-TV	Lys, 0.010(2); His, 0.003(1); Asp, 0.006(1); Thr, 0.012(2); Glu, 0.007(1); Pro, 0.010(2); Val, 0.003(1); Leu, 0.003(1)
C-18a-TVI	Lys, 0.007(2); Asp, 0.005(1); Ser, 0.004(1); Glu, 0.009(2); Pro, 0.004(1); Gly, 0.008(2); Ala, 0.008(2); Val, 0.003(1); Tyr, 0.004(1); Phe, 0.004(1)

TABLE IX

Tryptic and acid hydrolytic fragments of Peptide C-22

Tryptic fragment	Amino acid composition
C-22-TI (R_{FC}, 0.10; R_{FE}, 1.08)	Lys, 0.034(2); Thr, 0.010(1)[a]
C-22-TII (R_{FC}, 0.31; R_{FE}, 0.61)	Lys, 0.022(1); Asp, 0.033(1), Glu, 0.027(1); Ala, 0.025(1); Val, 0.028(1); Met, 0.006(1)[a]
C-22-TIII (R_{FC}, 0.13; R_{FE}, 0.67)	Lys, 0.014(2);[a] Arg, 0.009(1); Asp, 0.012(1); Glu, 0.036(3); Gly, 0.013(1); Val, 0.012(1); Ile, 0.010(1); Phe, 0.009(1)
C-22-TIV[b] (R_{FC}, 0.29; R_{FE}, 0.53)	Lys, 0.032(1);[a] Asp, 0.040(1); Thr, 0.013(1);[a] Tyr, 0.026(1); Glu, 0.014(0);[b] Gly, Ala, Val, Ile <0.007
Acid fragments	
C-22-AI[b] (R_{FC}, 0.20; R_{FE}, 0.78)	Lys, 0.13(2); Thr, 0.007(1); Glu, 0.008(1); Gly, 0.006(0);[b] Val, 0.007(1); Met, 0.006(1)
C-22-AII[b] (R_{FC}, 0.35; R_{FE}, 0.54)	Lys, 0.017(2);[a] Glu, 0.023(2); Gly, 0.012(0);[b] Ala, 0.007(1);[a] Val, 0.013(1); Ile, 0.009(1); Phe, 0.011(1)
C-22-AIII (R_{FC}, 0.08; R_{FE}, 0.80)	Lys, 0.023(1); Arg, 0.020(1); Thr, 0.022(1); Glu, 0.030(1); Gly, 0.021(1); Val, 0.004(0)
C-22-AIV (R_{FC}, 0.63; R_{FE}, 0.69)	Lys, 0.025(1); Tyr, 0.029(1)

[a] Partial destruction by ninhydrin staining is assumed.

[b] The residue numbers are assigned on a qualitative basis because of the rather large contamination with glutamic acid or glycine.

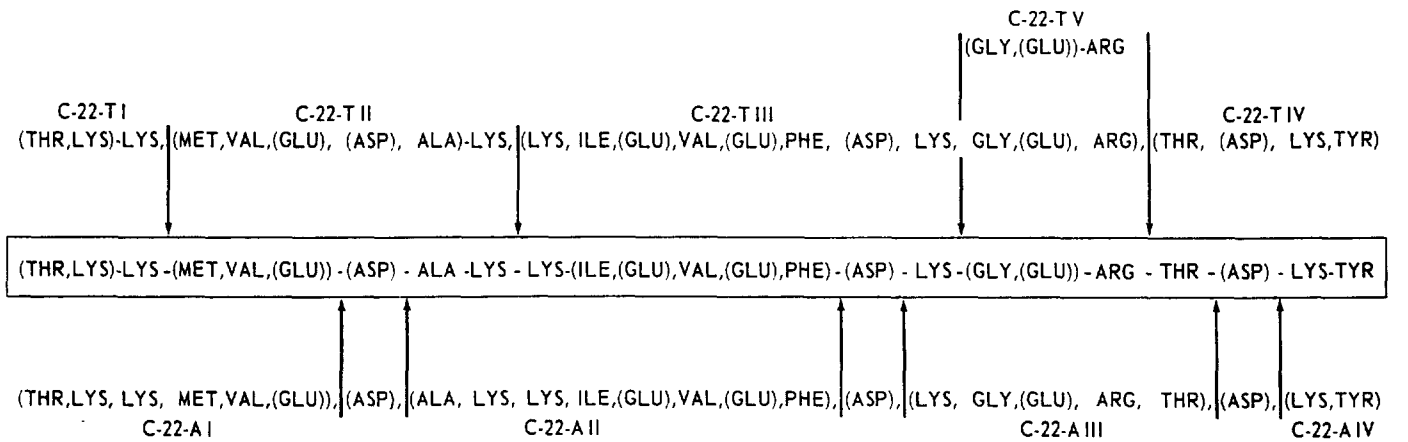

FIG. 5 Partial sequence of Peptide C-22. The *arrows shown above and below the partial sequence* indicate the peptide bonds cleaved by trypsin and dilute acid, respectively.

TABLE X

Partial sequences of chymotryptic peptides

Peptides marked with asterisks are used for the summation of the amino acid residues (see the text).

Peptide	Sequence
C-1	Ser-Glu-Asn (Asp, Ala) Asp-Ser (Gly, Gln)
C-2	Gly-Pro-Glu-Ala-Ser-Ala-Phe
C-4	Thr-Phe*
C-5d	(Lys, Asp, Gly, Ala) Met
C-5e	((Asp), Ala, Gly) Lys ((Asp), (Glu), Ala, Val, Met, Leu)*
C-5g[a]	Ala-Tyr
C-6a[a]	Val-Tyr*
C-6b[b]	Ala-Thr (Ser, Thr) Lys
C-7e[a]	Met-Tyr*
C-9c	Ile-Lys (Ala, Ile) ((Asp), Gly, (Asp), Thr) Val-Lys-Leu*
C-9d	(Lys, Gly, Pro, Gln) Met*
C-10b[c]	(Lys, Arg, Asp_2, Glu_2, Gly_2, Ala_2, Val_2, Met, Leu_2)
C-14a	Lys-Pro-Asn (Thr, Asn) (His, Glu, Gln) Leu*
C-14c	Ala-Lys (Ala, Val) Tyr*
C-15a	His-Lys ((Glu), Pro, Ala, Thr) Leu
C-15b	(Val, Arg) (Gln, Gly) Leu*
C-15c	Ala (Lys, Thr_2, Ser) Lys-Leu*
C-15e	Gly (Arg, Gly) Leu*
C-17a	(Asp)-Lys (Gly, (Glu)) Arg (Thr, (Asp), Lys) Tyr
C-17b[a]	(Lys, His, Asp_2, Thr, Glu_2, Pro, Val, Leu, Tyr)
C-18a	Leu-Val ((Asp), Thr, Pro, (Glu), Thr) Lys (His, Pro, Lys) (Lys, Gly, Val, (Glu)) Lys (Tyr, Gly, Pro, (Glu), Ala_2, Ser, Phe)*
C-19e	Gly (Arg, Gly, Leu, Ala) Tyr
C-19f	Arg-Leu-Leu*
C-20a	(Leu, Val, (Asp), Thr, Pro, (Glu), Thr) Lys (His, Pro, Lys) (Lys, Gly, Val, (Glu), Lys, Tyr)
C-20b[c]	(Lys_4, Arg, Ser, $(Glu)_3$, Ala_2, Leu) ($(Asp)_4$, Ser_2, $(Glu)_2$, Gly, Ala, Ile, Leu)*
C-22	(Thr, Lys) Lys (Met, Val, (Glu)) (Asp)-Ala-Lys-Lys (Ile, (Glu), Val, (Glu), Phe) (Asp)-Lys (Gly, (Glu)) Arg-Thr-(Asp)-Lys-Tyr*

[a] There is no special description of these peptides in the text (see Table III). However, the sequence of dipeptides was assigned on the basis of the specificity of chymotrypsin.

[b] The examination with Edman degradation and leucine aminopeptidase digestion indicated that this peptide was identical with tryptic Peptide T-V-8a.

[c] See the following paper (14).

are presented in Table VIII. Fragment C-18a-TIV, which did not contain lysine but contained both tyrosine and phenylalanine, was assigned to the COOH-terminal position of Peptide C-18a.

Digestion of Fragment C-18a-TI with leucine aminopeptidase released leucine and valine (Val, 0.013; Leu, 0.021). Combined digestion with carboxypeptidases A and B released only lysine from Fragment C-18a-TI.

The amino acid composition of Fragment C-18a-TVI included those of Fragments C-18a-TIII and C-18a-TIV. The amino acid composition of Fragment C-18a-TV (which included Thr and His) indicates that trypsin Fragments C-18a-TI and C-18a-TII constitute the NH_2-terminal portion of the parent Peptide C-18a.

C-19e: Gly (Arg, Gly, Leu, Ala) Tyr—Tyrosine was assigned as the COOH-terminal residue on the basis of the specificity of chymotrypsin. The yellow color developed by cadmium ninhydrin staining indicated an NH_2-terminal glycine residue.

C-19f: Arg-Leu-Leu—This peptide was assigned 1 residue of arginine and 2 of leucine (Table III). Carboxypeptidase A digestion (5 hours) released leucine (yield, 79%) and arginine (Leu, 0.034; Arg, 0.008). These results are consistent with the above sequence, assuming that the COOH-terminal leucine residue was completely released and that the resulting dipeptide, Arg-Leu, was 58% hydrolyzed.

C-20a: (Leu, Val, (Asp), Thr, Pro, (Glu), Thr) Lys (His, Pro, Lys) (Lys, Gly, Val, (Glu), Lys, Tyr)—Tryptic digestion yielded several fragments separated by paper electrophoresis: C-20a-TI, (Leu, Val, Asp, Thr_2, Pro, Glu, Lys); C-20a-TII, (His, Pro, Lys_2); and an overlapping fragment, C-20a-TIII.

Fragment C-20a-TI: Lys, 0.017(1); Asp, 0.015(1); Thr, 0.028(2); Glu, 0.014(1); Pro, 0.017(1); Val, 0.023(1); Leu, 0.016(1).

Fragment C-20a-TII: Lys, 0.016(2); His, 0.002(1); Pro, 0.006(1).

Fragment C-20a-TIII: Lys, 0.014(2); His, 0.007(1); Asp, 0.009(1); Thr, 0.016(2); Glu, 0.012(1); Pro, 0.021(2); Val, 0.008(1); Leu, 0.005(1).

C-22: (Thr, Lys) Lys (Met, Val, (Glu)) (Asp)-Ala-Lys-Lys (Ile, (Glu), Val, (Glu), Phe) (Asp)-Lys (Gly, (Glu)) Arg-Thr-(Asp)-Lys-Tyr—Neither leucine aminopeptidase nor carboxypeptidase A produced free amino acids from this peptide. Trypsin digestion of this peptide gave four fragments: C-22-TI, C-22-TII, C-22-TIII, and C-22-TIV. Acid hydrolysis with 0.03 N HCl provided four fragments: C-22-AI, C-22-AII, C-22-AIII, and C-22-AIV. These fragments were purified by two-dimensional peptide mapping, and their amino acid compositions are presented in Table IX. The summed amino acid residues of the four tryptic fragments accounts for the amino acid composition of Peptide C-22. Fragments formed by acid hydrolysis served to connect the tryptic fragments in order from the NH_2-terminal through the COOH-terminal residues as follows: Fragment C-22-AI (Lys_2, Thr, Glu, Val, Met) connected Fragment C-22-TI to C-22-TII. Fragment C-22-AII (Lys, Glu, Gly, Val, Ile, Phe) provided the connection between Fragments C-22-TII and C-22-TIII. Fragment C-22-AIII (Lys, Arg, Thr, Glu, Gly) bridged Fragments C-22-TIII and C-22-TIV.

Tryptic Fragment C-22-TV (R_{FC}, 0.19; R_{FE}, 0.79) showed the qualitative amino acid composition (Gly, Glu, Arg) and is included in Fragment C-22-TIII. Fragment C-22-TIV, containing tyrosine, should be the COOH-terminal fragment of peptide C-22, and Fragment C-22-TI the NH_2-terminal tryptic fragment. Fragment C-22-AIV (Lys, Tyr) may be assigned as part of Fragment C-22-TIV.

The relationships of the fragments, together with the deduced partial sequence of C-22, are presented in Fig. 5. Amide groups were not determined.

Relationship of Chymotryptic Peptides to Covalent Structure of Nuclease

The partial sequences of the chymotryptic peptides are summarized in Table X. Some of the isolated chymotryptic peptides were tentatively assigned to overlap other chymotryptic peptides on the basis of their partial sequences and chromatographic

yields, as summarized in Table X. The peptides marked with asterisks in Table X appear to account for the covalent structure of the nuclease without overlapping. The amino acid compositions of these peptides sum to give the following: Lys, 23; His, 3; Arg, 5; Asp, 14; Thr, 10; Ser, 5; Glu, 18; Pro, 6; Gly, 10; Ala, 14; Val, 9; Met, 4; Ile, 4; Leu, 12; Tyr, 6; Phe, 3. This composition is very similar to that obtained on the basis of the tryptic peptides, except for small differences in the contents of lysine, isoleucine, tryrosine, and tryptophan. The amino acid summation indicates that the chymotryptic peptides isolated cover essentially the entire amino acid sequence of the nuclease and serve to establish the linear arrangement of tryptic peptides described in the following paper (14).

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